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Research Article

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Screening of Urine and Blood Using Limited Sample Preparation and Information Dependent Acquisition with LC-MS/MS as Alternative for Immunoassays in Forensic Toxicology

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Abstract

Immunoassays are widely used to perform an initial toxicological screening of biological samples. However, LC-MS/MS is described as a promising technique that can overcome the limitations of immunoassays (such as their lack of selectivity). The objective of this project was to implement a LC-MS/MS method for screening of forensic ante- and post-mortem urine and whole blood samples that can replace the immunoassays. Easy and rapid sample preparation techniqueswere evaluated. Protein precipitation with acetonitrile combined with aqueous dilution (dilution factor 5 for urine and 10 for blood) proved to be an effective procedure. On the LC-MS/MS, 1 scheduled multiple reaction monitoring transition for each of 414 compounds was analyzed in positive mode, followed by an enhanced product ion scan if the peak height exceeded a specified threshold. In negative ionization mode, 38 compounds were measured with a scheduled multiple reaction monitoring method. For analysis of THC and two metabolites, a separate positive multiple reaction monitoring method was used to enhance sensitivity. 162 forensic urine samples and 146 blood samples were analyzed with both LC-MS/MS and immunoassay screening. LC-MS/MS screening was superior and can be considered as a trustworthy alternative to immunoassays in forensic toxicology.

Keywords

Forensic; Toxicology; Screening; Urine; Whole blood; LC-MS/MS; Immunoassay

Abbreviations

CE: Collision Energy; DAD: Diode Array Detection; EPI: Enhanced Product Ion Scan; GC: Gas Chromatography; LC: Liquid Chromatography; LLE: Liquid-Liquid Extraction; IDA: Information Dependent Acquisition; IS: Internal Standard; ME%: Matrix Effects; MRM: Multiple Reaction Monitoring; (MS/)MS: (tandem) Mass Spectrometry; RE%: Recovery; RT: Retention Time; sMRM: scheduled Multiple Reaction Monitoring; SPE: Solid-Phase Extraction; PE%: Process Efficiency

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Introduction

Immunoassays are widely used for screening of biological samples. In case of a positive result, an additional selective confirmation analysis is performed. Immunoassays are simple and quick, but costly. Moreover, immunoassays are not selective: no individualistic compound (e.g. diazepam), but only a group (e.g. benzodiazepines) is detected. Besides the compounds included in a group, other structurally related compounds can result in a false positive test because of cross-reactivity. Not all drug classes are covered by the immunoassays and some systems will disappear from the market (e.g. Abott Axsym® which is routinely used for screening of forensic (i.e. both ante- and post-mortem) urine samples in our laboratory). Therefore, we searched for an alternative for the immunoassays for screening of forensic urine and whole blood samples.

Two promising approaches using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for screening are described: multi-target screening and general unknown screening [1-6]. In the first approach only a selected group of compounds is detected, while this is not limited in the second approach. As a consequence, sensitivity of the multi-target screening is in general higher, but the number of compounds that can be detected is lower. For multi-target screening, a triple quadrupole, ion trap or a hybrid instrument combining these two are preferred as the mass analyzer. For general unknown screening, measuring the accurate mass of compounds using a time-of-flight mass analyzeris becoming more and more popular.

Several simple sample preparation methods for screening with LC-MS/MS are described [1-6]. For urine, dilution with different dilution factors and solvents, protein precipitation with several precipitating agents and filtration are used as simple alternatives for more complex liquid-liquid extraction (LLE) and solid-phase extraction (SPE) [1-5]. Screening of whole blood (especially post-mortem) is more difficult than urine or serum/plasma because of the complexity of this matrix. This explains why almost all LC-MS/MS methods for screening of whole blood use SPE or LLE as sample preparation [1-3]. There is only one paper describing a protein precipitation procedure (including an evaporation step) for screening of post-mortem whole blood [6]. Clearly, research on easy and quick sample preparation for whole blood is very limited. Moreover, to the best of our knowledge, there is no publication that describes LC-MS/MS screening of both ante- and post-mortem urine and whole blood.

The objective of this project was to develop an easy, quick and low-cost sample preparation and LC-MS/MS method for screening of forensic urine and whole blood samples.

Materials and Methods

Chemicals, standards and samples

CON-DOA®containing known concentrations of amphetamine, benzoylecgonine, codeine, dextropropoxyphene, methamphetamine, methadone, methaqualone, morphine, oxazepam and phencyclidine was purchased from Siemens Medical Solutions Diagnostics (LA, USA). The internal standard (IS) N-methylclonazepam was purchased from LGC (Molsheim, France). Acetone was purchased

from Merck (Darmstadt, Germany). Water was obtained from a Milli Q Water Purification System (Millipore, Brussel, Belgium). LC-MS grade acetonitrile was purchased from Biosolve (Valkenswaard, The Netherlands). All LC-MS grade mobile phase additives (formic acid and ammonium formate) were purchased from Sigma-Aldrich (Bornem, Belgium). Glassware was silanized using AquaSil Siliconizing Fluid (Thermo Scientific, Breda, The Netherlands). 1.5 mL screw cap vials, 100 µL inactivated glass vial inserts, Whatman® Mini-UniPrep™ syringeless filters with 0.2 µm or 0.45 µm PTFE filtration membranes and Toxitubes® were purchased from Agilent (Diegem, Belgium). Statistical analyses were performed with GraphPad Prism (version 6.01, La Jolla, US).

Immunoassays

For screening of urine samples, a fluorescence polarization immunoassay (Abbott Axsym® system, Waver, Belgium) was used to detect amphetamine/metamphetamine, barbiturates, benzodiazepines, cannabinoids, cocaine and metabolites, methadone, opiates and tricyclic antidepressants. Used cutoffs were 50 ng/mL for cannabinoids and tricyclic antidepressants, 60 ng/mL for benzodiazepines, 100 ng/mL for methadone, 150 ng/mL for cocaine, 200 ng/mL for barbiturates and opiates, 500 ng/mL for (met) amphetamine. Screening of blood samples was performed with an enzyme immunoassay (Cozart® system, Oxfordshire, UK) which detected the presence of amphetamine/metamphetamine, barbiturates, benzodiazepines, cannabinoids, cocaine and metabolites, methadone and opiates. Used cutoffs were 5 ng/mL for methadone, 10 ng/mL for cannabinoids, 20 ng/mL for barbiturates, 50 ng/mL for cocaine and metabolites, 100 ng/mL for (met)amphetamine, benzodiazepines and opiates.

Sample preparation for LC-MS/MS screening

Seven sample preparations were tested for screening of urine and blood: (1,2) precipitation with acetonitrile/acetone and aqueous dilution; (3,4,5,6) protein precipitation with acetonitrile/acetone, aqueous dilution and 0.2/0.45 µm filtration and (7) a simple LLE. For the precipitation procedures, 200 µL precipitating solvent (acetonitrile or acetone containing 500 ng/mL N-methylclonazepam) was slowly dropped to 100 µL supernatant of urine (150 µL urine was centrifuged for 10 min, 1000 × g) or blood while vortexing. After centrifugation (10 min, 1000 × g) the supernatant was diluted with 700 or 300 µL water (equal to a dilution factor 10 or 5) in a 1.5 mL vial or in the chamber of a Whatman® Mini-UniPrep™ syringeless filter (in this case volumes were decreased by half as the maximum volume of the filter is 500 µL). The plunger containing the 0.2 µm or 0.45 µm PTFE filtration membrane was manually pressed through the diluted sample into the chamber and the filtrate is forced into the reservoir of the plunger. The Mini-UniPrep filter was then placed in the autosampler of the LC-MS/MS. Compared to the common use of a syringe and filters, this syringeless system reduces waste and avoids contamination. For the LLE, the Toxitubes (Toxitubes A for extraction of basic and neutral drugs and Toxitubes B for extraction of acidic and neutral drugs) were vortexed for 10 s. Next, 100 µL urine or blood, 200 µL IS (500 ng/mL N-methylclonazepam in H_2O) and 4.7 mL water (in Toxitube A) or 4.2 mL water (in Toxitube B) were added. The tubes were mixed for 5 min and centrifuged (5 min, 1000 × g). The organic layer was evaporated to dryness at room temperature. The sample was reconstituted with 200 µL acetonitrile and 800 µL water and transferred into a 1.5 mL vial (equal to a dilution factor 10).

LC-MS/MS

LC-MS/MS analysis was carried out using an UFLC Shimadzu system consisting of 2 LC-20ADXR pumps, a SIL-20ACXR autosampler, a DGU-20A3 degasser and a CTO-20A oven (Shimadzu Prominence, Antwerpen, Belgium) in combination with a 3200 QTRAP (ABSciex, Halle, Belgium) and Analyst software (version 1.5). An existing multi-target screening approach for this kind of apparatus was adapted from the literature: a scheduled multiple reaction monitoring-information dependent acquisition-enhanced product ion (sMRM-IDA-EPI) multi-target screening approach [4]. The method starts with a survey sMRM scan, where MRM transitions are only monitored during the expected retention time window. When a sMRM signal exceeds a preset IDA-threshold, an EPI scan is performed (this is a product scan where the third quadrupole is used as a linear ion trap). However, the original sMRM-IDA-EPI screening method is only performed in positive ionization and situations occur where compounds cannot be identified because the MRM signal is too low to trigger an EPI scan or because the EPI quality is insufficient for identification [4]. The existing method was optimized to overcome these shortcomings. The number of detected compounds was reduced from 700 to only 414 forensic relevant compounds (Table 1). An extra sMRM method in negative ionization mode measuring two MRM transitions for 38 compounds was added in order to detect a broader range of compounds (Table 2). However, the positive and negative method were not sensitive enough for detection of cannabis use, as shown by preliminary comparison of LC-MS/MS and immunoassays for some real-life forensic samples of cannabis users. To enhance the sensitivity of the screening for detection of cannabis, an important drug in forensic investigations, a MRM method for analysis of THC, THC-OH and THC-COOH was added (Table 2). In summary, each sample was injected three times and analyzed by three LC-MS/MS methods: (1) a sMRM-IDA-EPI method in positive ionization mode, (2) a sMRM method in negative mode and (3) a positive MRM method for detection of THC, THC-OH and THC-COOH.

LC conditions

The used pentafluorophenyl propyl column (5.0 µm particle size, 2.1 mm x 50 mm), fitted with a guard column (2.1 mm × 10 mm, same packing material) and a filter of 2.0 µm, was purchased from Restek (via Interscience, Louvain-la-Neuve, Belgium). The autosampler temperature was set at 15°C, the column oven at 40°C. The autosampler needle was rinsed before and after sample injection to avoid carry over. The mobile phase consisted of water with 2 mM ammonium formate and 0.2% formic acid (A) and acetonitrile with 2 mM ammonium formate and 0.2% formic acid (B). The positive sMRM-IDA-EPI and negative sMRM method had following gradient conditions: 0-10 min: 10-90%B and increase of flow rate from 0.5 mL/min to 1 mL/min; 10-15 min: 90%B at 1 mL/min; 15-15.5 min: 90-10%B; 15.5-17.5 min: 10%B at 0.5 mL/min. Following gradient at a flow rate of 0.5 mL/min was used in the MRM method: 0-10 min: 10-90%B; 10-11 min: 90%B; 11-11.5 min: 90-10%B; 11.5-13.5 min: 10%B. The injection volume was 30 µL.

MS/MS conditions

For all three LC-MS/MS experiments, electrospray conditions were as follows: gas 1: nitrogen, 40 psi; gas 2: nitrogen, 70 psi; ion-spray voltage: 4000 V (-4000 V in negative mode); ion-source temperature: 500°C; curtain gas: nitrogen, 20 psi; collision gas: high. The declustering potential was 40 V (-40 V in negative mode), the entrance potential 10 V (-10 V in negative mode), the cell exit potential 5 V (-5

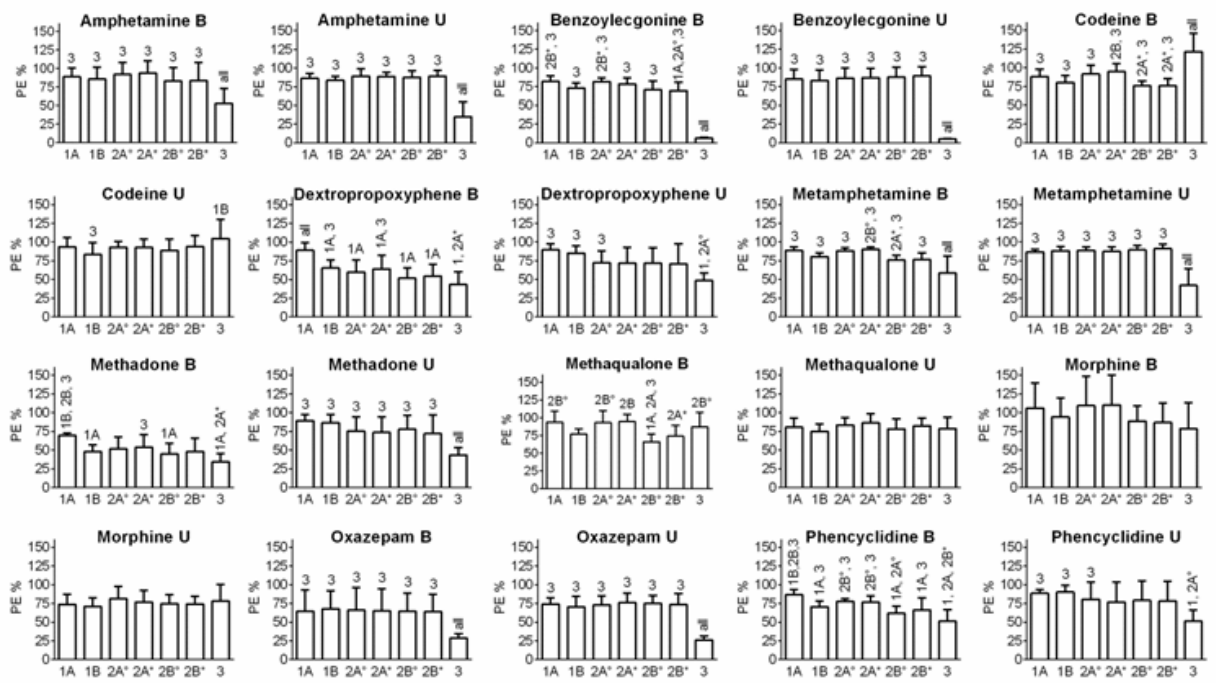

Figure 1: PE% of the different sample preparations tested for ten compounds in urine (U) and whole blood (B).
Tested methods included: precipitation with acetonitrile and 10-fold dilution (1A), precipitation with acetone and 10-fold dilution (1B), protein precipitation with acetonitrile, 10-fold dilution and 0.2 µm filtration (2A°), protein precipitation with acetonitrile, 10-fold dilution and 0.45 µm filtration (2A*), protein precipitation with acetone, 10-fold dilution and 0.2 µm filtration (2B°), protein precipitation with acetone, 10-fold dilution and 0.45 µm filtration (2B*) and LLE using the Toxitubes (equal to a dilution factor 10) (3). The average and standard deviation of 10 measurements (5 different sources of both urine and whole blood, analyzed in duplicate) are shown. The numbers of the sample preparations that are significantly different are noted above each sample preparation (one-way ANOVA, Tukey-Kramer test, $p<0.05$). Tested concentrations in urine and whole blood were 2.5 µg/mL amphetamine, 0.75 µg/mL benzoylecgonine, 1.5 µg/mL codeine, 0.375 µg/mL dextropropoxyphene, 2.5 µg/mL methamphetamine, 0.5 µg/mL methadone, 0.9 µg/mL methaqualone, 0.15 µg/mL morphine, 0.5 µg/mL oxazepam and 0.125 µg/mL phencyclidine.

V in negative mode). Q1 and Q3 were operated in unit resolution. In the sMRM-IDA-API method, 414 MRM are measured ±90 s around the expected retention time of the compound (Table 1). The target scan time was 1 s with a pause between the sMRM transitions of 2 ms. The used sMRM transitions were adapted from literature [4]. If the sMRM peak height exceeded the IDA-treshold (1000 counts per second (cps)) an EPI scan was triggered for the two most abundant sMRM signals. sMRM transitions which triggered the EPI scan twice consecutively were excluded for EPI scans for 15 s. The EPI scans were performed in a mass range from 50 to 640 Da at 4000 Da/s applying a collision energy (CE) of 35 ± 15 V, a fixed fill time of 50 ms and Q0 trapping. The acquired EPI spectra were automatically compared to the used ABSciex MS/MS library containing 1253 compounds [7]. In negative ionization mode, a sMRM method was used containing 2 sMRM transitions for each of 38 compounds (Table 2). Each MRM is measured ± 90 s around the expected retention time of the compound. For analysis of THC, THC-OH and THC-COOH a separate MRM method with dwell times of 30 ms was used (Table 2).

LC-MS/MS data analysis

For the sMRM-IDA-EPI method, a report was automatically generated. However, for accurate identification, manual review of the data is necessary [5]. Following criteria were used for identification: (1) the blank must be negative, (2) the IS must be present at the correct retention time with purity >75% and (3) the 4 (3 if ≤5 ions in the library spectrum) most abundant ions in the library spectrum must be present in the unknown spectrum. Ions present in the unknown spectrum that are not present in the library spectrum must be smaller than the 2 (3 if ≤5 ions in the library spectrum) most abundant ions in the unknown spectrum. For the (s)MRM methods, retention time, presence of the MRM transitions and ratio between MRM transitions were used for identification.

Method validation

The process efficiency (PE%) that includes the influence of possible matrix effects (ME%) and recovery (RE%) was calculated. Two sets of samples were prepared for determination of PE%. In set 1, blank matrices (5 different sources of both urine and whole blood) were spiked with pure standard before sample preparation. Set 2 consisted of pure standards. Fifteen compounds with varying characteristics representing the broad range of compounds detected in this screening were carefully selected. Following formula was used to calculate the PE%:

$$PE\% = \frac{ME\% \times RE\%}{100} = \frac{A}{B} \times 100\%$$

Where A is de peak area of the measured MRM transition from set 1, B from set 2. A value of 100% reflects the perfect situation. Clear guidelines on the acceptability of PE% values do not exist, so we had a look at the acceptance criteria for ME% and RE%. Acceptance criteria for ME% are set to 75-125% with a coefficient of variation

Table 1: Analytes measured in the positive sMRM-IDA-EPI method.

Analytes in positive sMRM-IDA-EPI			
17-Alpha-methyltestosterone	Clomipramine	Lisinopril	Pilocarpine
2-Amino-5-chlorobenzophenone	Clonazepam	Loperamide	Pindolol
2-Amino-5-nitrobenzophenone	Clonidine	Loratadine	Pioglitazone
2-Hydroxyethylflurazepam	Clopenthixol	Lorazepam	Pipamperone
3,4-Methylenedioxyamphetamine	Clopidogrel	Lormetazepam	Pirbuterol
3,4-Methylenedioxyethylamphetamine	Clozapine	Losartan	Pirenzepine
3,4-Methylenedioxymethamphetamine	Cocaine	Lysergide (LSD)	Piritramide
6-O-Monoacetylmorphine	Codeine	Maprotiline	Piroxicam
7-Aminoclonazepam	Coniine	MBDB	Prajmalium
7-Aminodesmethylflunitrazepam	Corticosterone	Mebeverine	Pramipexole
7-Aminoflunitrazepam	Cortisone	Meclizine	Prazepam
7-Aminonitrazepam	Cotinine	Medazepam	Prazosin
9-Hydroxyrisperidone	Coumatetralyl	Melatonin	Prednisolone
Acebutolol	Cyclicine	Melitracen	Prednisone
Aceclidine	Cytarabine	Meloxicam	Primidone
Aceclofenac	Desalkylflurazepam	Melperone	Procainamide
Acepromethazine	Desipramine	Mepindolol	Procyclidine
Aciclovir	Desmethylclobazam	Mescaline	Progesterone
Ajmaline	Desmethylclomipramine	Mesoridazine	Promazine (IS)
Alizapride	Dexamethasone	Metaclazepam	Promethazine
Allopurinol	Dexamethasone 21-isonicotinate	Metamfepramone	Prometryn
Alpha-hydroxyalprazolam	Dextromethorphan	Methamphetamine	Propafenone
Alpha-hydroxytriazolam	Dextropropoxyphene	Metformin	Propionylpromazine
Alprazolam	Diazepam	Methadone	Propranolol
Alprenolol	Diclofenac	Methaqualone	Propyphenazone
Amantadine	Diflucortolone	Methotrexate	Prothipendyl
Ambroxol	Dihydrocodeine	Methylephedrine	Protriptyline
Amiloride	Dihydroergotamine	Methylphenidate	Pseudoephedrine
Aminodantrolene	Dilazep	Metoprolol	Psilocin
Aminophenazone	Diltiazem	Metronidazole	Quetiapine
Aminopromazine	Dimetotiazine	Mexiletine	Quinapril
Amiodarone	Diphenhydramine	Mianserin	Quinine
Amiphenazole	Dipyridamole	Miconazole	Ramipril
Amisulpride	Disopyramide	Midazolam	Ranitidine
Amitriptyline	Dixyrazine	Midodrine	Reboxetine
Amlodipine	Dobutamine	Milrinone	Repaglinide
Amoxicillin	Doxapram	Minoxidil	Reserpine
Amphetamine	Doxepin	Mirtazapine	Risperidone
Apomorphine	Doxylamine	Mizolastine	Ritodrine
Aprindine	Ecgoninemethylester	Moclobemide	Ropinirole
Atenolol	EDDP	Modafinil	Ropivacaine
Atorvastatin	Embutramide	Molsidomine	Rosiglitazone
Atropine	Enalapril	Morphine	Salbutamol
Atropinemethylbromide	Ephedrine	Morphine 3-β-D-glucuronide	Scopolamine
Aztreonam	Eprosartan	Moxonidine	Serotonin
Baclofen	Esmolol	Nalorphine	Sertindole
Beclomethasone dipropionate	Estazolam	Naloxone	Sertraline
Befunolol	Ethenzamide	Naltrexone	Sildenafil
Bendiocarb	Etomidate	Nandrolone	Simazine
Benperidol	Etoposide	Naphazoline	Sotalol
Benzatropine	Felbamate	Nebivolol	Stanozolol
Benzocaine	Felodipine	Nicardipine	Sulfamethoxazole
Benzoctamine	Fenarimol	Nicotinamide	Sulindac
Benzoylecgonine	Fendiline	Nicotine	Sulpiride
Berberine	Fenethylline	Nifedipine	Sumatriptan
Betamethasone 21-phosphate	Fenfluramine	Nifenazone	Tadalafil
Betaxolol	Fentanyl	Nimodipine	Talinolol
Bezafibrate	Fexofenadine	Nisoldipine	Tamoxifen

Biperiden	Flecainide	Nitrazepam	Telmisartan
Bisoprolol	Fluconazole	Nitrendipine	Temazepam
Brodifacoum	Flumazenil	Norbuprenorphine	Tenoxicam
Bromazepam	Flunarizine	Nordiazepam	Terbinafine
Bromocriptine	Flunitrazepam	Norephedrine	Terbutaline
Brompheniramine	Fluoxetine	Norfenefrine	Terfenadine
Brotizolam	Flupentixol	Norfentanyl	Tetracaine
Bucetin	Fluphenazine	Norfloxacin	Tetrazepam
Bumetanide	Flurazepam	Normorphine	Tetryzoline
Bunitrolol	Fluvoxamine	Nortriptyline	Thebacon
Bupivacaine	Gabapentin	Noscapine	Theobromine
Bupranolol	Galantamine	Ofloxacin	Theophylline
Buprenorphine	Gallopamil	Olanzapine	Thiamine
Buspirone	Gemcitabine	Ondansetron	Thioridazine
Butaperazine	Glibenclamide	Opipramol	Tiagabine
Caffeine	Glibornuride	Ornidazole	Tiapride
Candesartan	Gliclazide	Orphenadrine	Ticlopidine
Captopril	Glimepiride	Oxazepam	Tilidine
Carazolol	Glipizide	Oxcarbazepine	Timolol
Carbamazepine	Gliquidone	Oxetacaine	Tizanidine
Carbamazepine 10,11-epoxide	Guaifenesin	Oxilofrine	Tocainide
Carbinoxamine	Haloperidol	Oxitropium	Tolbutamide
Carbuterol	Heroin	Oxprenolol	Toliprolol
Carteolol	Hydrocodone	Oxycodone	Tramadol
Carvedilol	Hydrocortisone	Oxymorphone	Tranexamic acid
Celiprolol	Hydrocortisone 21- acetate	Papaverine	Trazodone
Cetirizine	Hydromorphone	Paracetamol	Triamterene
Chlorcyclizine	Hydroxyzine	Paraoxon	Triazolam
Chlordiazepoxide	Imipramine	Paroxetine	Trifluperazine
Chlorphenethiazine	Indinavir	Pentamidine	Trifluperidol
Chlorpheniramine	Indapamide	Pentoxyverine	Triflupromazine
Chlorpromazine	Indometacin	Perazine	Trimethoprim
Chlorpromazine sulfoxide	Indoprofen	Perindopril	Trimipramine
Chlorprothixene	Ipratropium	Perphenazine	Urapidil
Cilazapril	Irbesartan	Phenelzine	Valsartan
Cinnarizine	Isoprenaline	Pheniramine	Vardenafil
Ciprofloxacin	Kavain	Phenprocoumon	Venlafaxine
Cisapride	Ketamine	Phenylephrine	Verapamil
Citalopram	Ketoprofen	Phenyltoloxamine	Vincamine
Clarithromycin	Ketorolac	Phenytoin	Warfarin
Clemastine	Lamotrigine	Pholedrine	Xylometazoline
Clenbuterol	Lercanidipine	Pethidine (Meperidine)	Yohimbine
Clobazam	Levocabastine	Phenacetin	Zolpidem
Clobenzepam	Levodopa	Phenazone	Zopiclone
Clobutinol	Levomepromazine	Phencyclidine	Zuclopenthixol
Clomethiazole	Lidocaine	Physostigmine	

Compound dependent parameters and retention times were adapted from literature [4].

(CV%= standard deviation divided by average) of maximum 15% (or 20% near the limit of detection) [8]. RE% is acceptable if the CV% is smaller than 15% (or 20% near the limit of detection) [8]. However, for qualitative methods acceptance criteria could be less strict. Selectivity was evaluated with blank samples from different sources (n=10 each for both urine and blood, no IS was added during sample preparation) and zero samples (n=2, IS was added to blank samples during sample preparation). To determine carryover, 30 µL H_2O was injected as blank after every sample. Finally, 162 forensic urine and 146 whole blood samples were analyzed using the LC-MS/MS method and compared with results from immunoassays and confirmation analyses, describing the accuracy, selectivity, sensitivity and carryover of the sample preparation and LC-MS/MS method. With each batch, a quality control sample (blood or urine spiked with CON-DOA) was run to check overall system performance.

Results and Discussion

Seven sample preparations (resulting in a 10-fold dilution of the urine and blood samples) were tested for ten compounds with varying characteristics representing the broad spectrum of compounds that can be found in forensic samples (amphetamine, benzoylecgonine, codeine, dextropropoxyphene, methamphetamine, methadone, methaqualone, morphine, oxazepam and phencyclidine) (Figure 1). Precipitation, aqueous dilution and filtration had an equal or lower

PE% than the same procedure without filtration (Figure 1(2 versus 1)). The LLE using the Toxitubes was highly variable and showed a low PE% for most of the compounds (Figure 1(3)). Samples treated with acetonitrile had in general less variability than samples treated with acetone (Figure 1(A versus B)). There was no significant difference between filtration membranes of 0.45 µm and 0.2 µm (Figure 1(* versus °)). Based on the following three criteria (i.e. PE% around 100%, low variability and ease of use), protein precipitation with acetonitrile and aqueous dilution was selected as the optimal sample preparation (Figure 1(1A)).

Since urine samples are in general cleaner and have a lower viscosity than whole blood samples, we tried to lower the dilution factor in order to enhance sensitivity. We tested undiluted urine, dilution factor 5 and 10 for the screening of spiked urine samples (see values for "urine, high", Table 3). As expected, significant and highly variable matrix effects were seen when directly injecting urine after centrifugation. Moreover, a shift in retention time from run to run was seen as a consequence of contamination of the LC column. Five- and 10-fold dilution had better PE% values and lower variability. As a compromise between sensitivity and repeatability, protein precipitation with acetonitrile and 5-fold dilution was selected as the sample preparation for urine.

The PE% of the selected sample preparations (i.e. protein precipitation with acetonitrile and 5-fold dilution for urine and protein precipitation with acetonitrile and 10-fold dilution for blood) was further measured in both matrices for the ten compounds at different concentration levels, to ensure its analytical quality at different concentrations and in blood for five substances almost 100%

Table 2: Analytes, their retention time (RT) and MRM parameters (Q1, Q2 and collision energy (CE)) measured in the negative sMRM method for detection of 38 compounds and the positive MRM method for detection of THC and metabolites.

Analyte (RT in min shown for sMRM)	Q1 (m/z)	Q2 (m/z)	CE (V)
2-Amino-5-nitrobenzophenone (4.3)	241.1	134.1/163.1	-35/-20
4-Benzamidosalicyclic acid (3.2)	256.1	212.1/134.0	-20/-35
5-Aminosalicylic acid (0.1)	152.0	108.0/107.0	-20/-35
5-(p-Methylphenyl)-phenylhydantoin (3.2)	265.1	116.0/102.0	-35/-35
6-Mercaptopurine (0.1)	151.0	92.0/90.0	-35/-50
Acetazolamide (0.5)	221.0	83.0/79.9	-35/-35
Acetylsalicylic acid (1.7)	179.0	93.0/137.0	-35/-20
Adenine (0.1)	134.1	107.0/92.0	-20/-35
Adenosine (0.1)	266.1	134.1/107.0	-50/-50
Adrenalone (0.1)	180.1	108.0/147.1	-35/-20
Alprostadil (3.1)	353.2	113.0/317.2	-50/-50
Amobarbital (2.6)	225.1	136.2/182.2	-35/-20
Amoxicillin (0.7)	364.1	206.1/93.0	-20/-35
Atorvastatin (4.4)	557.3	278.2/397.52	-50/-35
Aztreonam (0.1)	434.1	95.9/79.9	-50/-50
Barbital (0.0)	183.1	140.1/84.9	-20/-20
Benserazide (0.1)	256.1	137.0/109.0	-20/-35
Benzthiazide (3.3)	430.0	308.1/228.1	-35/-50
Bezafibrate (3.8)	360.1	274.2/154.1	-20/-35
Brallobarbital (2.9)	285.0	78.8/205.1	-20/-20
Butalbital (0.0)	223.1	180.2/94.1	-50/-20
Butallylonal (0.0)	301.0	78.9/221.2	-20/-35
Carbenoxolone (5.2)	569.4	469.3/99.0	-50/-50
Chlorothiazide (0.9)	293.9	214.0/179.1	-35/-50
Cimetidine (0.9)	251.1	97.0/123.1	-35/-20
Cortisone (2.5)	359.2	329.3/137.1	-20/-50
Dinoprost (3.1)	353.2	83.0/165.2	-35/-35
Epinephrine (0.1)	182.1	164.1/122.0	-20/-35
Ethylglucuronide (0.1)	221.1	84.9/74.9	-22/-22
Ethylsulfate (0.1)	125.0	96.8/79.8	-22/-42
Furosemide (2.9)	329.0	285.1/205.1	-20/-35
Hydrochlorothiazide (1.0)	296.0	205.1/77.9	-35/-50
Pentobarbital (2.6)	225.1	182.2/138.2	-20/-20
Phenobarbital (1.9)	231.1	188.2/144.1	-20/-20
Propallylonal (2.9)	287.0	78.8/207.1	-35/-20
THC-COOH (5.1)	343.2	299.3/245.2	-35/-36
THC-OH (5.5)	329.1	311.0/268.0	-27/-33
Triamcinolone (1.9)	393.2	345.3/325.3	-20/-20
THC	315.2	193.2/259.2/123.1	35/20/35
THC-OH	331.2	313.2/193.2/201.2	20/35/35
THC-COOH	345.2	327.2/299.2/193.2	20/20/35

A RT of 0.0 indicates that the MRM transitions are measured during the entire run (for compounds with an unknown RT)

bound to plasma proteins (see values in italic, Table 3). These five (buprenorphine, clomipramine, cocaine, midazolam and zolpidem) were included to ensure that the used sample preparation works for all compounds. The lowest PE% can be expected in blood, where the protein precipitation is more drastic than in urine. Drugs that are highly bound to proteins in the blood have an increased risk of being lost in the precipitant. Therefore, their behavior was tested. All PE% values were higher than 60% with CV% smaller than 30%, except for morphine in urine (see values in italic, Table 3). The obtained PE% values were considered acceptable for screening. The method was found to be selective. No carryover was seen.

Finally, the optimized sample preparation and three LC-MS/MS methodswere used for screening of 162 forensic urine samples and 146 whole blood samples (both ante- and post-mortem species). These samples were also analyzed with the appropriate immunoassay and confirmation techniques routinely used in the laboratory (Table 4). To evaluate LC-MS/MS as an alternative tool to immunoassay screening, the number of true and false positive and negative results obtained

Table 3: Fraction bound to plasma protein (Fb), PE% and CV% for 15 compounds in urine and blood analyzed with the LC-MS/MS screening.

Analyte (Fb)	Precipitation and 10-fold dilution						Precipitation and 5-fold dilution				Direct injection	
	Blood, low		Blood, high		Urine, high		Urine, low		Urine, high		Urine, high	
	PE%	CV%	PE%	CV%	PE%	CV%	PE%	CV%	PE%	CV%	PE%	CV%
Amphetamine (0.16)	*95*	*3*	*91*	*8*	86	8	*91*	*19*	*78*	*16*	48	41
Benzoylecgonine (?)	*87*	*8*	*82*	*5*	85	15	*69*	*21*	*79*	*20*	32	64
Buprenorphine (0.96)	*103*	*27*	*105*	*15*	-	-	-	-	-	-	-	-
Clomipramine (0.96)	*88*	*7*	*90*	*6*	-	-	-	-	-	-	-	-
Cocaine (0.92)	*115*	*7*	*113*	*2*	-	-	-	-	-	-	-	-
Codeine (0.07-0.25)	*104*	*6*	*96*	*11*	97	13	*66*	*27*	*75*	*15*	30	46
Dextropropoxyphene (0.78)	*89*	*12*	*95*	*5*	90	9	*101*	*7*	*100*	*11*	114	34
Methamphetamine (?)	*96*	*5*	*91*	*5*	87	4	*59*	*21*	*74*	*14*	33	53
Methadone (0.87)	*82*	*7*	*79*	*4*	89	9	*90*	*10*	*91*	*12*	176	34
Methaqualone (0.80)	*91*	*5*	*104*	*9*	80	15	*79*	*12*	*81*	*21*	44	53
Midazolam (0.96)	*93*	*8*	*88*	*6*	-	-	-	-	-	-	-	-
Morphine (0.35)	*104*	*14*	*100*	*17*	74	18	*56*	*44*	*60*	*27*	19	66
Oxazepam (0.87-0.94)	*78*	*8*	*79*	*6*	74	11	*82*	*15*	*64*	*25*	42	45
Phencyclidine (0.65)	*69*	*10*	*87*	*8*	89	6	*101*	*6*	*96*	*5*	96	26
Zolpidem (0.93)	*91*	*11*	*74*	*30*	-	-	-	-	-	-	-	-

Tested methods included: protein precipitation with acetonitrile and dilution with water (resulting in a dilution factor 5 or 10) and direct injection (after centrifugation). The average PE% and CV% of 10 measurements (5 different sources of both urine and whole blood, analyzed in duplicate) are shown. Tested concentrations were 0.3 and 2.5 µg/mL amphetamine, 0.09 and 0.75 µg/mL benzoylecgonine, 0.1 and 1 µg/mL buprenorphine, 0.18 and 1.5 µg/mL codeine, 0.1 and 1 µg/mL clomipramine, 0.1 and 1 µg/mL cocaine, 0.05 and 0.375 µg/mL dextropropoxyphene, 0.3 and 2.5 µg/mL methamphetamine, 0.15 and 0.5 µg/mL methadone, 0.1 and 0.9 µg/mL methaqualone, 0.1 and 1 µg/mL midazolam, 0.06 and 0.150 µg/mL morphine, 0.05 and 0.5 µg/mL oxazepam, 0.015 and 0.125 µg/mL phencyclidine and 0.1 and 1 µg/mL zolpidem in urine and blood, low and high concentrations respectively. The values in italic show the PE% and CV% of the selected optimal sample preparation.

Table 4: Real-life forensic urine and blood samples screened with immunoassays and LC-MS/MS.

Urine samples	**AXSYM® immunoassay**				**LC-MS/MS screening**				**Confirmation**	
	False +	**False -**	**True +**	**True -**	**False +**	**False -**	**True +**	**True -**	**+**	**-**
Amphetamine/methamphetamine	6	1	14	141	1	1	14	146	15	147
Barbiturates	2	0	2	158	0	0	2	160	2	160
Benzodiazepines	2	24	52	84	0	8	65	86	76	86
Cannabinoids	2	4	21	135	0	1	24	137	25	137
Cocaine	1	12	12	137	0	5	19	138	24	138
Methadone	21	1	13	127	0	2	12	148	14	148
Opiates	1	13	13	135	1	4	22	135	26	136
Tricyclic antidepressants	9	0	3	150	0	0	3	159	3	159
TOTAL	44	55	130	1067	2	21	164	1109	185	1111
Blood samples	**Cozart® immunoassay**				**LC-MS/MS screening**				**Confirmation**	
	False +	**False -**	**True +**	**True -**	**False +**	**False -**	**True +**	**True -**	**+**	**-**
Amphetamine/methamphetamine	4	6	3	133	1	1	8	136	9	136
Barbiturates	0	0	1	145	0	0	1	145	1	145
Benzodiazepines	0	32	24	90	0	11	45	90	56	90
Cannabinoids	0	5	16	125	0	2	19	125	21	125
Cocaine	0	12	5	129	0	4	13	129	17	129
Methadone	0	0	3	143	0	0	3	143	3	143
Opiates	0	12	8	126	0	5	15	126	20	126
Total	**4**	**67**	**60**	**891**	**1**	**23**	**104**	**894**	**127**	**895**

The number of total positive (+) and negative (-) samples was derived from the information obtained with confirmation techniques (GC-MS, LC-DAD, LC-MS/MS). 75% of the analyzed urine samples and 57% of the blood samples were post-mortem. 47 of 162 urine samples and 63 of 146 blood samples were negative for all compound classes analyzed by the immunoassays.

by both techniques was compared. For urine samples, screening by immunoassay resulted in a higher number of false positives (44 versus 2) and false negatives (55 versus 21) than screening by LC-MS/MS (Table 4). For blood samples, the immunoassay also had a higher number of false negatives (67 versus 23) and false positives (4 versus 1) (Table 4). The Cozart® immunoassay had a lower number of false positives than the Axsym® system, as the Cozart® tests include less compounds in each class. The higher number of false positives for urine samples analyzed by the immunoassay can be explained by the low selectivity of the immunoassay: there is cross-reactivity with molecules structurally related to the target analyte(s) (e.g. in post-mortem samples, amphetamine-like compounds can be present because of putrefaction, generating a false positive result in the immunoassay). The higher number of false positives results in a higher cost (since more confirmation tests are required). False negative results were seen for samples containing low levels of drugs or if a compound is just not detected by the immunoassay (e.g. the Cozart® test for opiates does not react with fentanyl or tramadol which are detected by LC-MS/MS). Evidently, false negatives should be avoided in forensic toxicology. Considering the number of true and false results, LC-MS/MS screening was more specific, sensitive and correct than the immunoassay. The saving of time of screening by LC-MS/MS compared to the immunoassays was limited because of the need to analyze each sample with three different LC-MS/MS methods. However, because of the gain in efficiency, analysis time was considered of secondary importance.

Conclusions

In the search for an alternative for screening by immunoassays, several sample preparations of urine and whole blood followed by LC-MS/MS analysis were evaluated. Protein precipitation using acetonitrile combined with aqueous dilution (dilution factor 5 for urine and 10 for blood) proved to be an effective sample preparation for screening using LC-MS/MS. The samples were analyzed by three LC-MS/MS methods (sMRM-IDA-EPI for 414 positively charged compounds, negative sMRM for 38 acidic compounds and positive MRM method for THC and two metabolites) in order to cover a broad range of forensic relevant compounds. To the best of our knowledge this is the first LC-MS/MS method for screening of both ante- and post-mortem urine and whole blood. For 162 forensic urine samples and 146 whole blood samples, screening by LC-MS/MS performed superiorly compared to the immunoassays. Hence, LC-MS/MS can be considered a trustworthy alternative to immunoassays for screening in forensic toxicology.

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